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4-(5,6-Dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide as a fluorescent labelling reagent for determination of carboxylic acids in precolumn high-performance liquid chromatography

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Abstract

A fluorescent labelling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide, was designed for the determination of carboxylic acids by precolumn HPLC. The reagent was reacted with carboxylic acids at 100°C for 15 min in the presence of an activating reagent (a mixture of triphenylphosphine, 2,2′-dipyridyldisulfide and pyridine) and produced highly fluorescent derivatives, which were separated on a reversed-phase column by fluorescence measurement at 317 nm (excitation) and 380 nm (emission). The detection limits were 4–12 fmol/injection. The reagent was used for HPLC assays of long chain fatty acids in human serum by isocratic elution.

Keywords: Derivatization, LC; Detection, LC; 4-(5,6-Dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide; Carboxylic acids; Fatty acids

1. Introduction

The determination of carboxylic acids which are of physiological and clinical importance, requires a sensitive and selective detection technique. For sensitive HPLC determination of carboxylic acids which absorb only weakly in the UV–vis region and possess no native fluorescence, derivatization techniques using suitable labelling reagents are used. A variety of labelling reagents having chromophores, fluorophores and electrophores have been developed. Among these reagents, fluorogenic labelling reagents are preferred for detection of carboxylic acids at trace levels. The fluorescence labelling reagents have both a fluorophore group and a reactive functional group for carboxylic acids, the reactive functional groups are divided into the following types; bromomethyl-, bromoacetyl-, diazomethyl-, sulphonate-, hydrazide-, isourea-, alcohol- and alkylamine- [1–3]. The reagents having alkylamines as a reactive functional group, i.e. dansyl semipiperazide (DNS-Pz) [4], monodansyl cadaverine (MDC) [5], 2-[*p*-(5,6-methylenedioxy-2H-benzotriazol-2-yl)]phenethylamine [6], 4-(aminosulphonyl)-7-(1-piperazinyl)-2,1,3-benzoxadiazole (ABD-Pz) [7], 4-(N,N-dimethylaminosulphonyl)-7-N-piperadino-2,1,3-benzoxadiazole (DBD-Pz) [8], N-(4-aminobutyl)-N-ethylisoluminol [9], 4-aminomethyl-6,7-dimethoxycoumarin (ADC) [10] and 4-N,N-dimethyl-aminosulphonyl)-7-N-(2-aminoethyl)aminobenzofurazan (DBD-ED) [11] react with carboxylic acids to form the corresponding fluorescent amides in the presence of

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activation agents such as N,N-dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), diphenylphosphoryl azide (DPPA), diethyl phosphorocyanide (DEPC), triphenylphosphine–2,2′-dipyridyldisulfide–pyridine (Mukaiyama-A) or 2-chloro-1-methylpyridiniumiodide–triethylamine (Mukaiyama-B). These labelling reagents, although they require activating reagents, seem to be suitable for selective determinations of carboxylic acids in biological samples because functional groups other than the carboxylic acid group do not react with these alkylamine-type reagents.

We have previously reported developing the fluorescent labelling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl chloride (DPS-Cl), for determination of amine and amino acids and demonstrated that the derivatives (sulfonamides) of proline and hydroxyproline with DPS-Cl were highly fluorescent [12,13]. Therefore, the derivatives of secondary amines with DPS-Cl are considered to be useful as a fluorophore for labelling reagents. Herein, we report the synthesis of 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide (DPS-Pz) as a fluorescent labelling reagent for determination of carboxylic acids by HPLC and its application to the assay of fatty acids in human serum.

2. Experimental

2.1. Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. DPS-Cl, commercially supplied by Dojinndo (Kumamoto, Japan), was prepared as described previously [12]. Deionized, distilled water was purified with Milli-QII system (Yamato, Tokyo, Japan) prior to use. Lauric acid, myristic acid, oleic acid, 2,2′-dipyridyl disulfide and 2-chloro-1-methylpyridinium iodide were purchased from Tokyo Kasei (Tokyo, Japan), linolenic acid, stearic acid, triethylamine and DEPC were from Wako (Osaka, Japan) and linoleic acid, palmitic acid, piperadine anhydrous, triphenylphosphine, EDC and DPPA were from Nacalai Tesque (Kyoto, Japan). These chemicals were used as received. Mukaiyama-A was prepared by addition of pyridine (142 μl) to an acetonitrile solution (5 ml) containing triphenylphosphine (20 m*M*) and 2,2′-dipyridyl disulfide (20 m*M*) and Mukaiyama-B was prepared by addition of triethylamine (72 μl) to an acetonitrile solution (5 ml) containing 2-chloro-1-methylpyridinium iodide (20 m*M*).

2.2. Synthesis of and analytical data for 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl semipiperazide (DPS-Pz)

The mixture of piperazine anhydrous (1.1 g, 12.8 mmol) and DPS-Cl (470 mg, 1.28 mmol) in chloroform (125 ml) was stirred overnight at room temperature. The solution was washed with water (150 ml, twice), and the organic layer evaporated to dryness under reduced pressure. The residue dissolved in chloroform was chromatographed on a silica gel column (Wako Gel C-200, 150×30 mm, prepared with chloroform) with chloroform, followed by methanol to collect the second blue fluorescent band. This fraction was evaporated to dryness. The residue was recrystallized from ethanol to obtain DPS-Pz.

DPS-Pz: white needles (yield 271 mg, m.p. 241–243°C). Analysis: calculated for $C_{20}H_{23}N_3O_5S$, C 57.54, H 5.55, N 10.07%; found, C 56.95, H 5.67, N 9.77%. MS(CI, *m*/*z*): 418 ($[M+H]^+$). ^{1}H-NMR (δ, ppm, in C^2HCl_3): 2.98 (8H, s, CH_2 of piperazine), 3.98 and 4.00 (3H each, s each, OCH_3), 4.80 (2H, s, CH_2 of phthalimidine), 6.99 and 7.36 (1H each, s each, aromatic H of phthalimidine), 7.77 and 8.05 (2H each, d each, J=9.02 Hz each, H of benzene ring).

2.3. Preparation of and analytical data for the DPS-Pz derivatives of palmitic acid (DPS-Pz-Pa)

To a solution of palmitic acid (60 mg) in dichloromethane (25 ml), EDC (90 mg) in dichloromethane (25 ml) and DPS-Pz (100 mg) in dichloromethane (100 ml) were added and refluxed for 1 h. The mixture was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column (Wakogel C-200, 150×30 mm I.D.) with benzene–acetone (70:30, v/v) as eluent. The first fluorescent fraction collected was evapo-

rated to dryness and the residue recrystallized from methanol to obtain DPS-Pz-Pa.

DPS-Pz-Pa: white plates (yield 90 mg, m.p. 183–185°C). Analysis: calculated for $C_{36}H_{53}N_3O_6S$, C 65.92, H 8.14, N 6.41%; found, C 65.94, H 8.19, N 6.35%. MS (CI, *m/z*): 656 ($[M+H]^+$). ^{1}H-NMR (δ, ppm, in C^2HCl_3): 0.70~2.36 (31H, m, $C_{15}H_{31}CO$-), 2.77~3.16 and 3.46~3.83 (4H each, m each, CH_2 of piperazine). Other signals appeared at the same chemical shifts as those of DPS-Pz.

2.4. Instrumental conditions

The HPLC system consisted of a CCPS HPLC pump (Tosoh, Tokyo, Japan), a CO-8020 column oven, an SD-8022 on-line degasser, an AS-8020 auto-sampler, an FS-8020 fluorescence detector, and an SC-8020 super system controller (all from Tosoh). The separations were performed on a Symmetry C_8 column (150×3.9 mm I.D., 5 μm; Waters) connected with another Symmetry C_8 (20×3.9 mm I.D., Waters) as a guard column by means of isocratic elution with 10 m*M* phosphoric acid–acetonitrile (20:80, v/v) at 25°C. The flow-rate was 0.5 ml/min. The fluorescence intensities were monitored at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 317 and 380 nm, respectively. Uncorrected fluorescence spectra were measured with a Hitachi (Tokyo, Japan) Model 650-10S spectrofluorimeter using a quartz cell (optical path length, 10 mm).

2.5. Labelling procedure

To 100 μl of carboxylic acids in chloroform, 50 μl of Mukaiyama-A and a DPS-Pz reagent solution (2 m*M* in acetonitrile, 200 μl) were successively added and well mixed. The mixture was heated at 100°C for 15 min on a heating block. The reaction mixture was diluted 10-fold with acetonitrile–water (80:20, v/v) and an aliquot (20 μl) of the resulting mixture was injected into HPLC. For reaction conditions of carboxylic acids with DPS-Pz studies, a standard solution of four saturated fatty acids (lauric acid, myristic acid, palmitic acid and stearic acid) and three unsaturated fatty acids (linolenic acid, linoleic acid and oleic acid) each at a concentration of 10 μ*M* was employed.

2.6. Application to the determination of free fatty acids in serum

Free fatty acids in serum were extracted with a mixture of chloroform and *n*-heptane according to the conventional procedure [14]. Briefly, 50 μl serum was mixed with 200 μl 0.5 *M* phosphate buffer (pH 6.5) and then extracted with 500 μl chloroform–heptane (50:50, v/v). An aliquot (100 μl) of organic phase was placed in a stoppered test tube, and the labelling reaction described above carried out. After a 10-fold dilution of the reaction mixture with acetonitrile–water (80:20, v/v), an aliquot (20 μl) of the resulting mixture was injected into the HPLC.

3. Results and discussion

3.1. Structures and fluorescence spectra of DPS-Pz and DPS-Pz-PA

DPS-Pz, which is stable at room temperature and easily synthesized by the reaction of piperazine with DPS-Cl, was developed as a highly fluorescent labelling reagent for determination of carboxylic acids. DPS-Pz, which is itself fluorescent (λ_{ex} and λ_{em} in acetonitrile were 316 and 380 nm, respectively), reacted with carboxylic acids in the presence of activation agents to form corresponding fluorescent derivatives (Fig. 1). The structure of the DPS-Pz derivative of palmitic acid (DPS-Pz-Pa) was confirmed by elemental analysis, MS and ^{1}H-NMR spectral data.

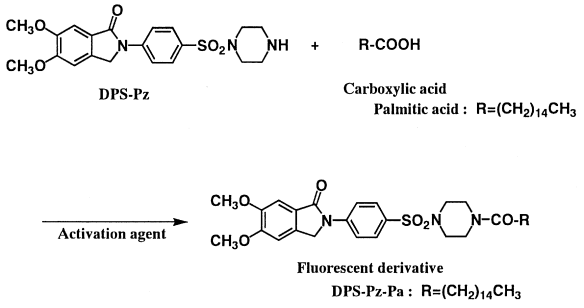

Fig. 1. Labelling reaction of carboxylic acid with DPS-Pz.

The fluorescence spectra of DPS-Pz-Pa in acetonitrile and aqueous acetonitrile were measured. The sample solutions were prepared by dilution of a chloroform solution of 1 m*M* DPS-Pz-Pa with acetonitrile to 20 μ*M* and then successively with water, aqueous acetonitrile (25, 50 and 75%) and acetonitrile to 1 μ*M*. The λ_{ex} and λ_{em} maximum wavelengths of DPS-Pz-Pa were 317 and 380 nm, respectively, (Fig. 2) regardless of the acetonitrile content in the medium, although the fluorescence intensity was affected by acetonitrile content. The fluorescence intensity in 50% aqueous acetonitrile was the most intense (Fig. 3). The pH of the medium was altered using 10 m*M* phosphoric acid and 10 m*M* phosphate buffer (pH 3, 5, 7 and 9) instead of water in a 50% aqueous acetonitrile solution, but the alterations did not affected either the fluorescence intensity or the maximum fluorescence wavelength.

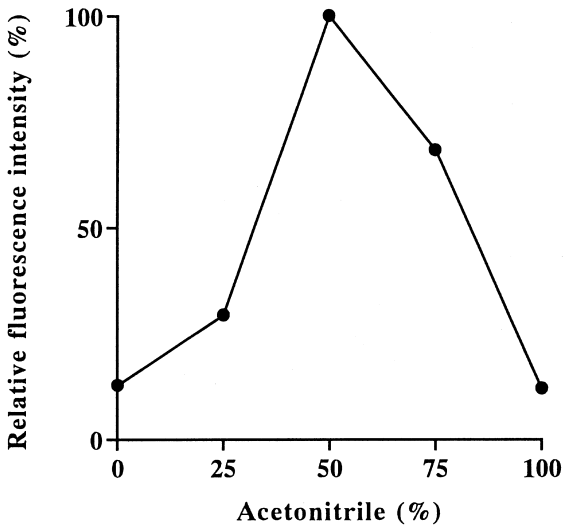

Fig. 3. Effect of acetonitrile content in the medium on fluorescence intensity of DPS-Pz-Pa.

3.2. HPLC separation

The derivatives of lauric acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid labelled with DPS-Pz were separated on a reversed-phase column with isocratic elution of 10 m*M* phosphoric acid–acetonitrile (20:80, v/v) within 25 min. A typical chromatogram is shown in Fig. 4. The peaks due to lauric acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid eluted at 6.8, 8.9, 9.6, 11.7, 14.4, 16.1 and 22.7 min, respectively, and were completely separated from the peaks of the reagent blank components. These peaks were identified by injections of derivatized single reference compounds, further, the retention time of the palmitic acid peak coincided with that of prepared DPS-Pz-Pa.

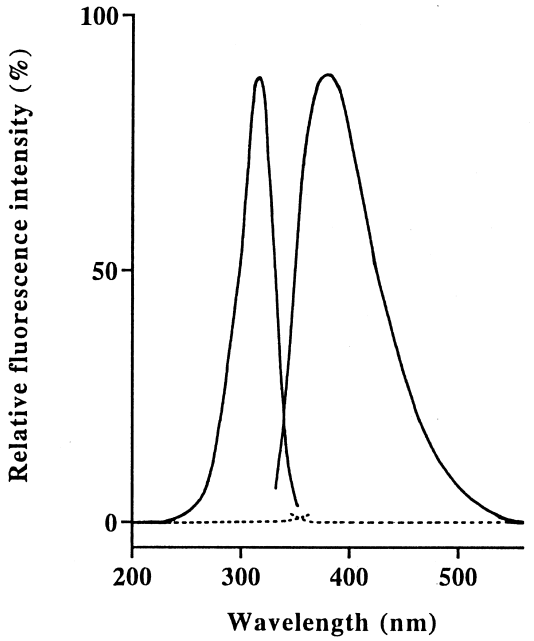

Fig. 2. Fluorescence spectra of DPS-Pz-Pa in 50% aqueous acetonitrile.

Phosphoric acid in the mobile phase affected the retention time of excess DPS-Pz present in the reaction mixture, whereas it did not affect those of the derivatives of carboxylic acids with DPS-Pz. Without phosphoric acid, the DPS-Pz eluted as a broad peak at 100 min. By the acidification of the reaction mixture or the mobile phase with phosphoric acid, hydrochloric acid or sulfuric acid, DPS-Pz eluted at 2–4 min. However, since the derivatives of carboxylic acids were unstable in the acidic medium (the peak area decreased to about 90% after 5 h), only the mobile phase was acidified with phosphoric acid.

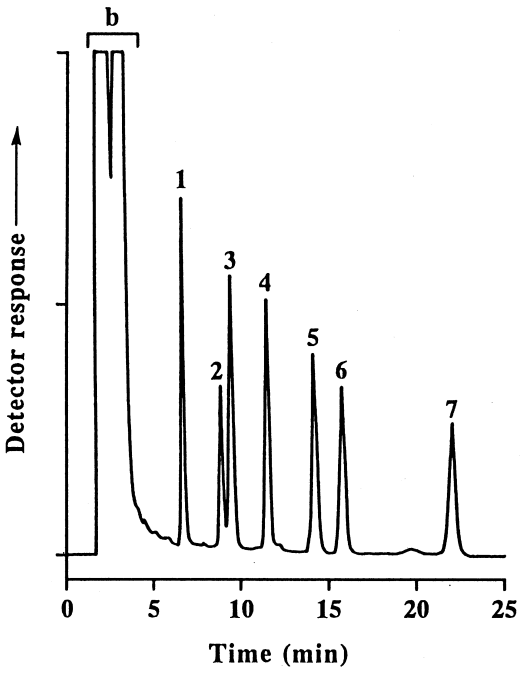

Fig. 4. Chromatogram obtained from a standard solution of seven carboxylic acids (10 μ*M* each) labelled with DPS-Pz. Peaks: 1=lauric acid; 2=linolenic acid; 3=myristic acid; 4=linoleic acid; 5=palmitic acid; 6=oleic acid; 7=stearic acid; b=reagent blank.

3.3. Reaction conditions

As the labelling reaction of carboxylic acids with DPS-Pz proceeded in the presence of activation agents, the effect of activation agents such as Mukaiyama-A or -B, EDC (20 m*M*), DPPA (20 m*M*) or DEPC (20 m*M*) were examined using a standard solution of lauric acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid (10 μ*M* each). The reaction was carried out in the test tube with a seal (100°C, 15 min) or without a seal (100°C, 30 min; the solvent was removed during the reaction). The maxima peak area of the fluorescent derivatives of carboxylic acids were obtained by the reaction at 100°C for 15 min with a seal using Mukaiyama-A as an activation agent. Peak areas relative to Mukaiyama-A (with a seal): Mukaiyama-B: 47.4–50.6%; EDC: 68.8–71.8%; DPPA: 25.5–26.0%; DEPC: 10.9–11.4% (with a seal); Mukaiyama-A: 86.7–92.5%; Mukaiyama-B: 42.9–46.8%; EDC: 68.4–72.9%; DPPA: 86.1–89.9%; DEPC: 86.2–92.8% (without a seal).

Reaction times were examined at various temperatures. The results obtained from palmitic acid are shown in Fig. 5. The peak area reached a maximum after reaction for 15 min at more than 80°C. Similar results were obtained for the other carboxylic acids. Consequently, the labelling reactions for determination of carboxylic acids with DPS-Pz were carried out at 100°C for 15 min.

The concentration of DPS-Pz in acetonitrile was determined by use of a standard solution of seven long chain carboxylic acids (10 and 50 μ*M* each). The most intense and constant peak areas were obtained at a reagent solution concentration of more than 1 m*M*.

The reaction mixture was stable for at least 24 h at room temperature. The efficiency of conversion of palmitic acid into the fluorescent derivative under the experimental conditions was examined by comparing the peak area with that of prepared DPS-Pz-Pa. The extent of conversion was 100.3%.

The effect of water on the labelling reaction was also examined. When the reaction was carried out in

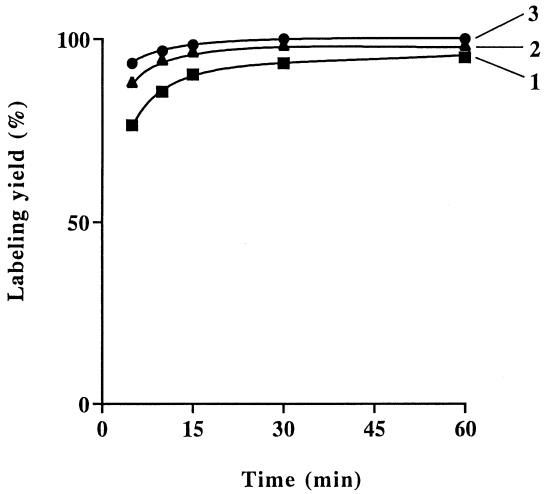

Fig. 5. Effect of reaction time and temperature on labelling reaction of palmitic acid with DPS-Pz. Curves: 1, 50°C; 2, 80°C; 3, 100°C.

the presence of water (10–200 μl), the small amount of water (10–100 μl) did not affect the reaction, although the large amount of water (200 μl) reduced the labelling yield to 83%.

3.4. Linearity, precision and detection limit

Peak areas were linear in relation to concentrations of the seven carboxylic acids over a wide range of concentrations (between 0.2 and 50 μ*M*). Peak precision was examined using ten replicate assays of a standard solution of carboxylic acids (5 μ*M*). The relative standard deviations of lauric acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid were 2.5, 6.0, 3.9, 1.8, 2.6, 1.3 and 2.5%, respectively. The detection limit (signal-to-noise ratio =3) for the carboxylic acids was 4–12 fmol per injection. This value is lower (more sensitive) than DNS-Pz (1000 fmol) [2] and MDC (below 100 fmol) [5] and comparable with values obtained from methods using ABD-Pz (10–20 fmol) [7], DBD-Pz (3–14 fmol) [8], ADC (10–50 fmol) [10] and DBD-ED (4–7 fmol) [11].

3.5. Application to the determination of serum fatty acids

To assess the usefulness of DPS-Pz for biological sample analysis, long chain fatty acids in serum were measured using the procedures in Section 2. The carboxylic acids were extracted with a mixture of chloroform and heptane which did not affect the labelling reaction. However, the small peaks corresponding to saturated fatty acids were observed in the reagent blank when the reaction mixture was directly injected to HPLC without a dilution step. Therefore, a dilution step prior to HPLC analysis was employed to eliminate the influence of the reagent blank. Typical HPLC chromatograms obtained from a human serum sample and the reagent blank are shown in Fig. 6. The peaks due to the fluorescent derivatives of lauric acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid in human serum were identified by comparing the retention times with those of a standard solution and prepared DPS-Pz-Pa, and also by cochromatography of the standard solution and serum. The concentrations (mean±S.D.) of lauric

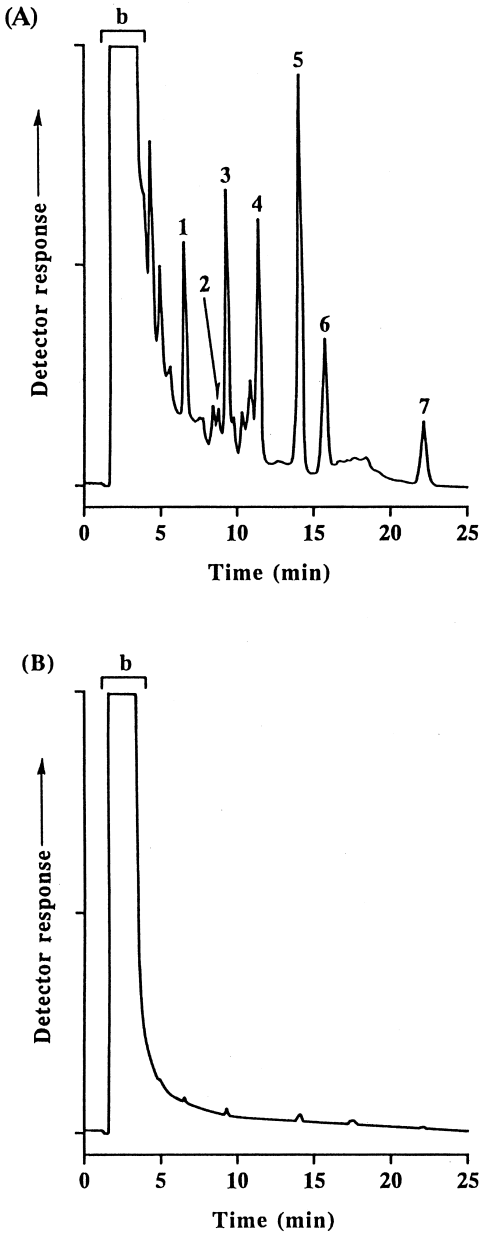

Fig. 6. Chromatograms obtained from (A) human serum and (B) reagent blank according to the procedure described in Section 2. Peaks (concentration): 1=lauric acid (14.8 μ*M*); 2=linolenic acid (3.6 μ*M*); 3=myristic acid (27.9 μ*M*); 4=linoleic acid (25.4 μ*M*); 5=palmitic acid (60.4 μ*M*); 6=oleic acid (23.6 μ*M*); 7=stearic acid (14.2 μ*M*).

acid, linolenic acid, myristic acid, linoleic acid, palmitic acid, oleic acid and stearic acid in serum from eleven healthy students in our laboratory were 10.7±4.3, 4.5±1.1, 38.4±21.0, 75.8±49.1, 160.3±77.3, 92.6±61.0 and 45.0±43.1 μ*M*, respectively.

4. Conclusions

In conclusion, we have synthesized DPS-Pz and shown it to be suitable as a fluorescent labelling reagent for the determination of carboxylic acids. Derivatives of carboxylic acids with DPS-Pz are stable and highly sensitive. Furthermore, DPS-Pz can be used to separate seven long chain fatty acids in serum on a reversed-phase column with isocratic elution within 25 min. Further studies on the application of DPS-Pz to biological samples — i.e. to real trace analysis, which is the ultimate test of every new reagent — are now in progress in our laboratory.

References

[1] Y. Ohkura, H. Nohta, Adv. Chromatogr. 29 (1989) 221.
[2] Y. Ohkura, M. Kai, H. Nohta, J. Chromatogr. B 659 (1994) 85.
[3] Y. Yasaka, M. Tanaka, J. Chromatogr. B 659 (1994) 139.
[4] I. Yanagisawa, M. Yamane, T. Urayama, J. Chromatogr. 345 (1985) 229.
[5] S. Narita, T. Kitagawa, Anal. Sci. 5 (1989) 31.
[6] Y.M. Lee, H. Nakamura, T. Nakajima, Anal. Sci. 5 (1989) 681.
[7] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Imai, Analyst 116 (1991) 609.
[8] T. Toyo'oka, M. Ishibashi, Y. Takeda, K. Nakashima, S. Akiyama, S. Uzu, K. Imai, J. Chromatogr. 588 (1991) 61.
[9] O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J.J.M. Holthuis, A.K. Smilde, D.A. Doornbos, J. Chromatogr. 615 (1993) 97.
[10] K. Sasamoto, T. Ushijima, M. Saito, Y. Ohkura, Anal. Sci. 12 (1996) 189.
[11] P. Prados, T. Fukushima, T. Santa, H. Homma, M. Tsunoda, S. Al-Kindy, S. Mori, Y. Yokosu, K. Imai, Anal. Chim. Acta 344 (1997) 227.
[12] H. Inoue, K. Moritani, Y. Date, K. Kohashi, Y. Tsuruta, Analyst 120 (1995) 1141.
[13] H. Inoue, Y. Date, K. Kohashi, H. Yoshitomi, Y. Tsuruta, Biol. Pharm. Bull. 19 (1996) 163.
[14] H. Tsuchiya, T. Hayashi, M. Sato, M. Tatsumi, N. Takagi, J. Chromatogr. 309 (1984) 43.